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In Vitro Comparisons of SENCAR and BALB/c Primary Epidermal Cells

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Grafting experiments show that the enhanced sensitivity of the SENCAR mouse to skin carcinogenesis by initiation and promotion is a property of the skin itself, suggesting the usefulness of *in vitro* studies to elucidate the mechanism. Such studies have indicated that cultured epidermal cells of SENCAR mice and the resistant BALB/c strain are remarkably similar in a variety of respects. DNA repair and carcinogen binding are quantitatively similar in cultured cells of SENCAR and more resistant mouse strains. Epidermal Langerhans cell (LC) number and LC-mediated functions were indistinguishable in SENCAR and BALB/c mice. Primary epidermal cells cultured in the presence of various concentrations of 12-O-tetradecanoylphorbol-13-acetate (TPA), retinoic acid, epidermal growth factor (EGF), hydrocortisone, or fluocinolone acetonide failed to reveal differences in growth between BALB/c and SENCAR cells. Cells from these animals bound comparable amounts of EGF with similar kinetics, and the modulation of this binding by TPA and retinoic acid was indistinguishable between strains. Spontaneous expression of infectious, endogenous xenotropic type C RNA virus at very low levels could be demonstrated in primary BALB/c epidermal cells and both BALB/c and SENCAR epidermal lines resistant to Ca^{2+}-induced terminal differentiation. The number of foci of initiated cells after exposure to carcinogens *in vivo* or *in vitro* did not differ significantly between SENCAR and BALB/c, suggesting that SENCAR sensitivity is primarily to promotion. However, there are qualitative differences between SENCAR and BALB/c foci. The appearance of foci of cells resistant to terminal differentiation in untreated SENCAR cultures supports the evidence from *in vivo* studies for the existence of a constitutively initiated cell population in SENCAR mouse skin.

Introduction

The SENCAR mouse was derived by selective breeding for rapid development of large numbers of papillomas after initiation with dimethylbenz(a)anthracene (DMBA) and promotion with 12-O-tetradecanoylphorbol-13-acetate (TPA) (*1*). Subsequent studies have shown that the enhanced sensitivity exists for initiators with a variety of different chemical structures, including the directly acting *N*-methyl-*N'*-nitro-*N'*-nitrosoguanidine (MNNG), a carcinogenic nitrosamide that does not require metabolic activation (*2*). Such data, along with a report that metabolites of DMBA are similar in epidermal homogenates of skin of SENCAR mice and the less sensitive parent CD-1 strain (*1*), suggest that the enhanced sensitivity of SENCAR mice does not result from differences in metabolism of polycyclic aromatic hydrocarbon carcinogens. Further evidence tending to rule out metabolic differences came from a study that showed the SENCAR mouse to be more highly sensitive to skin carcinogenesis after a single dose of ultraviolet (UV) radiation than the more resistant CD-1 strain (*3*).

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A major finding in elucidating the mechanism of SENCAR sensitivity was that the susceptibility is a property of the skin itself rather than a systemically mediated phenomenon (*4*). Skin of SENCAR mice and the resistant BALB/c mouse strain grafted to athymic nude mouse recipients retained the tumor incidence of the donor animals after initiation and promotion. This result validates the use of *in vitro* studies with cultured skin cells to elucidate the mechanism of SENCAR sensitivity and led to the work described in this paper. However, *in vivo* experiments have continued to be useful to define areas to be explored in culture and to confirm *in vitro* results. We have most frequently used BALB/c as our representative resistant strain for comparisons with the susceptible SENCAR mice since the work of Hennings et al. (*2*), using initiation-promotion *in vivo*, helped to define the basic biology of comparative epidermal carcinogenesis in these strains. In SENCAR mice, more papillomas and carcinomas developed with a shorter latency period than in BALB/c animals. This was true whether adult or newborn mice were used and whether DMBA or MNNG was used as an initiator, although DMBA was the stronger initiator. This study provided two additional pieces of possibly significant

information. First, SENCAR skin was notably more sensitive to the toxic effects of TPA than BALB/c skin. SENCAR mice developed ulcerative lesions, requiring the promoter to be applied less frequently. Second, SENCAR mice developed significant numbers of papillomas and carcinomas with TPA alone, in the absence of an exogenous initiator whereas BALB/c mice did not. This result may imply that SENCAR mouse skin contains a population of constitutively initiated cells. This hypothesis is supported by evidence from *in vitro* experiments to be discussed later in this paper.

Materials and Methods

Animals

SENCAR mice were obtained from the National Cancer Institute (NCI) Frederick Center Research Facility, Frederick, MD. BALB/c and other mouse strains were obtained through the National Institutes of Health (NIH) Small Animal Section, Bethesda, MD.

Cell Culture

Primary epidermal cells were obtained from newborn mice ($\leq$ 3 days old) by the trypsin flotation method as described previously (*5*) and cultured in Eagle's minimal essential medium (MA Bioproducts, Walkersville, MD) with 8% Chelex-treated fetal calf serum (Reheis Chemical Co., Kanakakee, IL). The final Ca^{2+} concentration in the medium was adjusted to 0.05 mM to select for basal cells as described by Hennings et al. (*6*). To induce terminal differentiation, the Ca^{2+} concentration was raised to 1.4 mM (*6*). Cell cultures were routinely incubated at 36.5°C in a humidified atmosphere of 7% CO_2 in air. For cell counts, cells were removed from plastic culture dishes by incubation with 0.06% trypsin, 0.1% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4 dissolved in H_2O to a total volume of 1 L) and counted in a Coulter counter.

Detection of Infectious Retrovirus

Virus detection was attempted in three ways: (1) from culture media in which the various cells were growing, (2) from cell homogenates, and (3) from intact epidermal cells by cocultivation with target cells. Two target cell lines were used: normal mink lung (CCL64) and an sarcoma-positive/leukemia-negative (S^+L^-) derivative of 313 mouse cells (D56). The former has previously been used to isolate mouse xenotropic type C retroviruses (*7*), and the latter has been shown to be a suitable cell line for propagation of both N- and B-tropic ecotropic murine retroviruses (*8*). Replicate cultures were inoculated with sterile medium as virus-negative controls. Target cells were passaged at 7-day intervals for 6 to 8 passages. Virus replication was determined by assaying for the presence in the culture fluid of sedimentable viral DNA polymerase (reverse transcriptase) activity (*9*) immediately before cell passage.

Immunological Assays

F_c-IgG receptors were determined by rosette assays (*10*) with sheep red blood cells, generously provided by Mr. Paul Becker of the National Institute of Neurological and Communicative Disorders and Stroke, and antiserum against sheep red blood cells (Cordis Laboratories, Miami, FL). Ia antigens were determined as previously described (*10*), with either polyspecific Ia antiserum (AT.H anti-A.TL) or with normal mouse serum as negative control, and the second antibody was fluorescein-isothiocyanate-conjugated rabbit anti-mouse IgG (Cappel Laboratories). The presence of keratin was similarly determined by indirect immunofluorescence microscopy using anti-keratin antisera kindly provided by Dr. Miriam Poirier, NCI. ATPase-positive cells were determined by the method of MacKenzie and Squier (*11*). Contact sensitivity to 2,4,6-trinitrochlorobenzene (Sigma Chemical Co., St. Louis, MO) was assessed as previously described (*12*). For the epidermal lymphocyte reaction, spleen cells from C57Bl/6 mice were used as responder T-cells. Primary epidermal cells to be tested were exposed to 2000 rads of X-irradiation and incubated with purified spleen T-cells. After 5 days, 10 μCi [^{3}H]thymidine (25 Ci/mmole, New England Nuclear, Boston, MA) were added to each well for the last 16 hr of culture.

Results and Discussion

DNA Repair and Carcinogen Binding

One of the most striking genetically based skin cancer susceptibility conditions in humans is xeroderma pigmentosum, in which the ability to repair DNA damaged by UV irradiation and chemical carcinogens is defective. Using the host cell reactivation assay previously used to characterize repair-defective xeroderma cell lines, we examined the ability of primary epidermal cells of SENCAR, CD-1, BALB/c, and AKR mice to repair UV-irradiated herpes simplex virus. These mouse strains form a spectrum of sensitivities to skin carcinogenesis from susceptible to resistant, respectively. Cells of all four strains were equally capable of reactivating irradiated virus (*13*). Furthermore, two nontumorigenic BALB/c epidermal cell lines resistant to Ca^{2+}-induced terminal differentiation responded similarly. In a related study, we found similar levels of DNA polymerase β, the putative repair enzyme, in primary epidermal cells from BALB/c and SENCAR mice (Strickland, unpublished observations). Others have shown that binding of the activated carcinogen *N*-acetoxyacetylaminofluorene to SENCAR and BALB/c primary epidermal cells exposed *in vitro* was quantitatively and qualitatively similar (Miriam Poirier, personal communication). These results suggest that susceptibility of SENCAR epidermal cells to carcinogenesis is probably not accounted for on the basis of alterations in DNA repair or carcinogen binding.

Table 1. Summary of immunological properties of epidermal cells from SENCAR and BALB/c mice.

	BALB/c[a]	SENCAR[a]
Langerhans cell density in epidermis, cell/mm^2		
ATPase staining	714 ± 153	696 ± 142
Ia antigen staining	383 ± 40	375 ± 88
Fc-positive cells in fresh epidermal cell suspensions, %	2.9	2.7
Alloantigen presenting ability[b]	3321 ± 384	3158 ± 265
Allergic contact sensitization, 10^{-2} × mm[c]	6.0 ± 1.2	4.9 ± 2.0

[a] All values are means ± standard errors of the mean.

[b] [^{3}H]thymidine incorporation by 2×10^5 T-cells from C57B1/6 mice cocultured 5 days with 5×10^4 irradiated BALB/c or SENCAR epidermal cells. Background was 689 ± 81 cpm.

[c] Increment in ear swelling on application of 1% 2,4,6-trinitrochlorobenzene after sensitization 6 days previously (corrected for negative control).

Immunological Factors

UV-radiation-induced skin carcinogenesis in mice has been shown to involve activation of suppressor T-cells that permit progressive growth of UV-induced tumors (*14*). In view of the results of carcinogenesis studies on grafted skin, we have examined two areas of immune responses that involve the epidermis. In collaboration with Dr. Thomas Luger of the NCI, we measured epidermal thymocyte activating factor (ETAF), which is produced by keratinocytes and interacts with T-lymphocytes. Under a variety of conditions—with and without TPA, in medium with high and low Ca^{2+} levels—SENCAR primary epidermal cell cultures produced amounts of ETAF similar to those found in BALB/c cultures (Lugar and Strickland, unpublished observations). Epidermal Langerhans cells (LC) are dendritic, suprabasal cells derived from bone marow that play a major role in cell-mediated immune functions of the skin, including allergic contact sensitivity and antigen and alloantigen presentation. We detected similar numbers of LC in epidermis and fresh epidermal cell suspensions of both newborn and adult BALB/c and SENCAR mice by several independent analytical methods. However, in primary newborn epidermal cell cultures examined 24 hr after plating, there were more than four times as many Fc-positive BALB/c cells as SENCAR. This difference probably results from SENCAR LC being less adherent to plastic than BALB/c LC, which suggests to us cell surface differences. Survival in culture of LC from the two strains was similar, with greatly enhanced survival in serum that had been heated at 56°C for 30 minutes before adding to the growth medium. We have been unable to associate any functional significance with the presumed LC cell surface differences. Functional studies in adults, including contact sensitization and alloantigen presenting ability, showed no significant strain differences. Table 1 summarizes these immunological results. Topical treatment of skin *in vivo* or of cultured primary epidermal cells with compounds having tumor-promoting activity such as TPA, teleocidin, and mezerein, lead to a marked increase in cells with Fc receptors (data not shown). Since these cells stain with antibody to Ia antigen but not with antikeratin antibody, we believe that tumor-promoter treatment results in actual increased numbers of LC rather than in induction of Fc receptors in other cell types. However, no significant strain differences have been found in the response of LC to tumor promoters. Therefore, we have been unable to implicate epidermal immune functions in the mechanism of SENCAR sensitivity to carcinogenesis.

In Vitro Growth Properties

We examined the growth kinetics of epidermal cells from BALB/c and SENCAR mice under high and low Ca^{2+} conditions, in response to TPA, retinoic acid, epidermal growth factor (EGF), hydrocortisone, and fluocinolone acetonide by cell counts as well as by thymidine incorporation measurements on a daily basis for a time period after treatment. Basically, significant strain differences were not seen in the effects of these agents on cell growth in culture (Fig. 1). Neither fluocinolone acetonide (Fig. 1*b*) nor hydrocortisone (not shown) at doses from 0.1 to 10 μg/mL affected proliferation of cells of either strain. Treatment with 100 ng/mL TPA led to an approximately 50% loss of attached cells in both strains, consistent with the induction of terminal differentiation in half the cells (*15*). Considerable emphasis was placed on study of EGF interactions with cultured epidermal cells because of the possibility of a transforming-type growth factor produced by SENCAR cells and the modulating effects of TPA and retinoic acid on EGF binding in other cell types. These studies are reported elsewhere (*16*). The only significant strain difference found was in the rate of loss of EGF binding capacity after induction of terminal differentiation by raising Ca^{2+} levels in the medium. The decrease was consistently faster and greater in SENCAR cells compared to BALB/c. There is no obvious relationship between this property and SENCAR susceptibility, however. Otherwise, BALB/c and SENCAR primary epidermal basal cells responded similarly as follows: over the range tested (10^4–10^5 cells/cm^2), EGF binding per cell increased two-to-three fold with increasing cell density. The two strains had similar numbers of receptors per cell. TPA treatment quickly (< 30 min) and strongly (80–90% at 100 ng/mL) suppressed the ability of cells to bind EGF (Fig. 2), whereas treatment of epidermal basal cells with retinoic acid resulted in increased EGF binding (Fig. 2). Treatment with EGF at 1 and 10 ng/mL stimulated cell growth and resulted in higher saturation densities compared to controls without EGF. Cell growth was stimulated only in proliferating cultures. It was ineffective, for example, if cells were plated at very low density. Binding curves indicated that 36 hr into the differentiation program induced by Ca^{2+}, cells bound lower levels and no longer metabolized EGF. Furthermore, differentiating cells were much less responsive to the modulation of EGF receptors by TPA and retinoic acid than were basal cells.

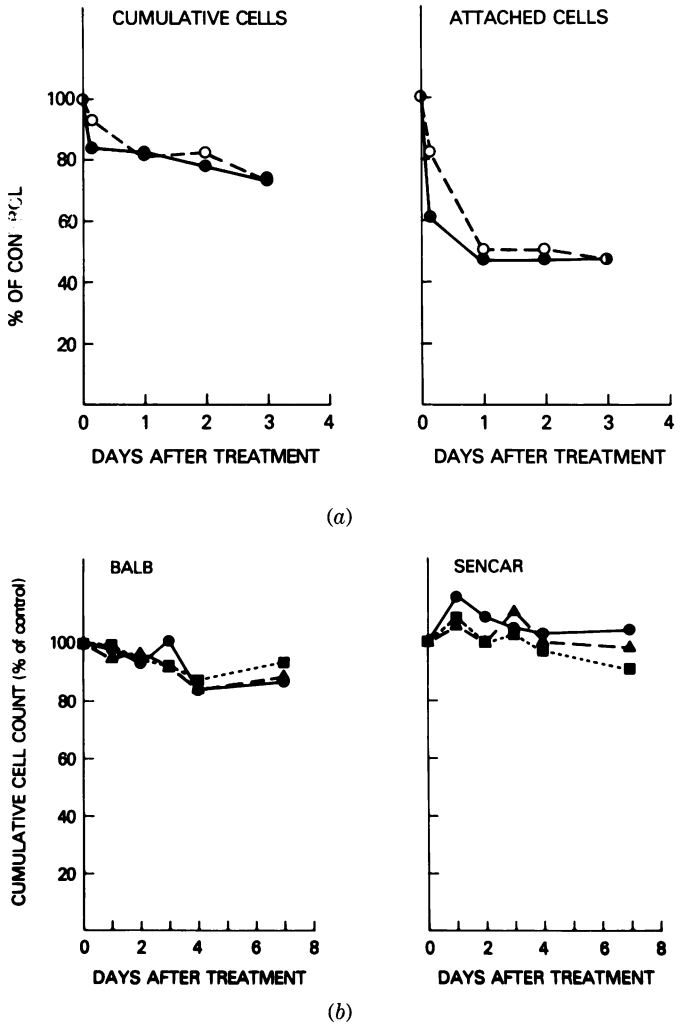

FIGURE 1. Growth of cultured primary newborn epidermal cells in the presence of (*a*) TPA or (*b*) fluocinolone acetonide. 2×10^6 cells were plated in 60-mm dishes. Medium with 0.05 mM Ca^{2+} was changed daily, and treatment was begun 6 days after plating with TPA or fluocinolone acetonide subsequently present throughout. Attached and unattached cells were counted at the times indicated. Cumulative cells were determined by summing the number of new cells each day with the number of attached cells at the beginning of treatment. Cumulative and attached BALB/c (●) and SENCAR (○) cells treated with 100 ng/mL TPA, expressed as % of solvent treated (0.1% dimethyl sulfoxide) controls; Cumulative cell counts of BALB/c and SENCAR cells exposed to 0.1 (●), 1.0 (▲), or 10 (■) μg/mL fluocinolone acetonide, expressed as % of solvent (0.1% DMSO) control.

Virus and Oncogene Expression

We found spontaneous expression of endogenous xenotropic but not ecotropic type C RNA virus in epidermal cell lines from both SENCAR and BALB/c mice. This expression occurred at low levels, since infectious virus was demonstrable only by cocultivation of epidermal cells with appropriate target cells and not in homogenates or culture fluid from these same cells. Furthermore, xenotropic virus expression was not seen in SENCAR primary cells but only in a cell line resistant to Ca^{2+}-induced terminal differentiation, whereas virus was detected in primary BALB/c epidermal cells as well as in lines with altered differentiation properties. Again, this virus was only detectible by cocultivation for 4 to 6 weeks with target cells. Epidermal cells from BALB/c and SENCAR mice had similar levels of surface receptor for gp 70, the major murine leukemia virus (MuLV) envelope glycoprotein, and were good target cells for infection with Rauscher and AKR MuLV. These results are summarized in Table 2. Although expression of infectious endogenous retrovirus is highly repressed, we have, in collaboration with W. K. Yang of Oak Ridge National Laboratory, detected virus-related sequences in poly A RNA preparations from primary epidermal cells as well as in lines derived from primaries by carcinogen treatment and selection for resistance to terminal differentiation (Yang and Strickland, unpublished observations). An RNA species with sequences homologous to the viral long terminal repeat (LTR), which contains elements that regulate gene expression, is expressed in several resistant cell lines but is absent from primary epidermal cells of both strains. The level of expression of a number of oncogenes did not differ significantly in SENCAR and BALB/c primary epidermal cells (Dennis Roop, NCI, personal communication). From the present data we cannot rule out expression of one or more of these oncogenes at normal levels but with an altered gene product in the cells tested.

Is SENCAR Sensitivity Related to Initiation or to Promotion?

A major achievement in defining the mechanism of SENCAR sensitivity to skin carcinogenesis would be to determine whether initiation, promotion, or both are involved. Promotion is clearly an important factor. The latent periods before the first papillomas and carcinomas appear in initiation-promotion studies is shorter, often by many weeks, in SENCAR than in resistant mouse strains. SENCAR skin is unusually sensitive to toxic effects of TPA. In the *in vivo* studies of Hennings et al. (*2*), SENCAR, but not BALB/c mice, developed ulcerative skin lesions in response to TPA, necessitating reduction of treatments from twice to once per week. SENCAR epidermal cells have no more phorbol ester receptors than resistant strains (Peter Blumberg, NCI, personal communication), nor have we been able to identify any unusual biochemical response of cultured SENCAR cells to phorbol ester tumor promoters. The study of Strickland (*3*), reporting sensitivity of SENCAR mice to skin tumor development after a single dose of UV radiation, suggested UV might provide a chronic promotion stimulus because irradiated skin of SENCAR mice, but not CD-1 controls, "appeared raised and leathery for several months" after irradiation. *In vivo* carcinogenesis studies from several laboratories have shown that mouse strains quite resistant to skin carcinogenesis by initiation-promotion may be very sensitive to tumor induction by multiple carcinogen treat-

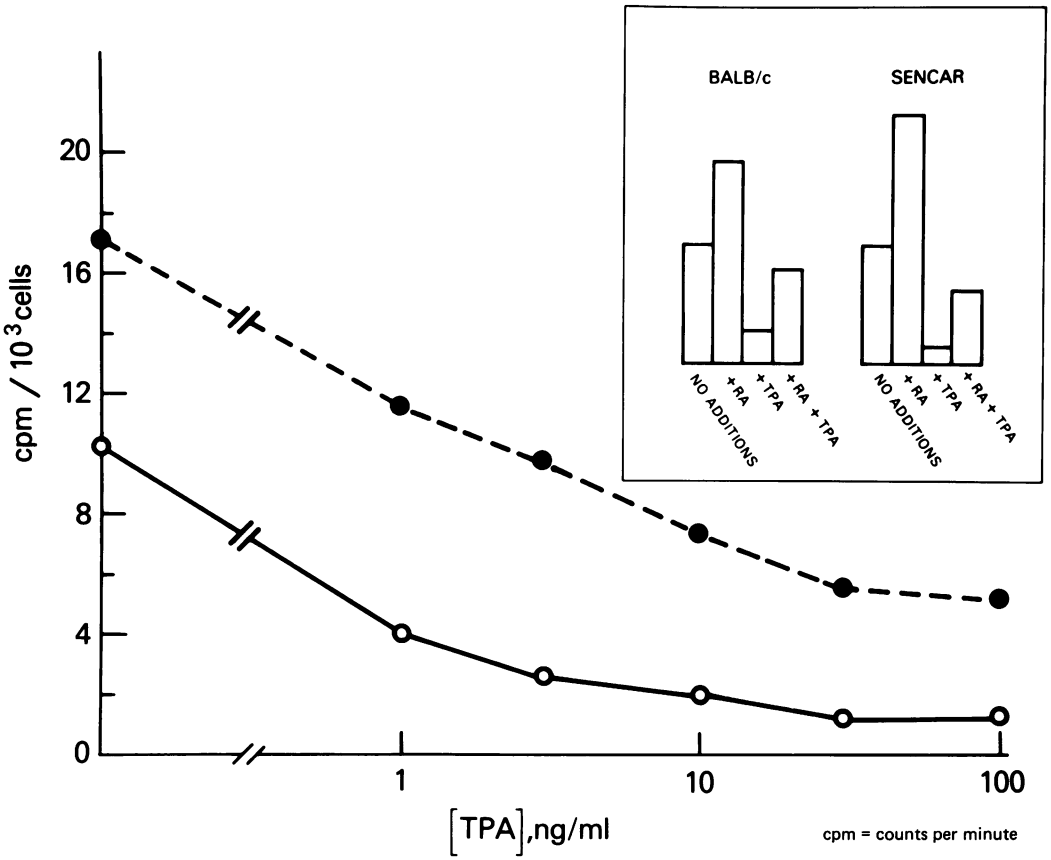

FIGURE 2. Dose-response curve for the reduction in EGF binding in BALB/c primary epidermal basal cells by treatment (2.5 hr at 37°C) with TPA in the presence (●) or absence (○) of pretreatment with retinoic acid (10^{-5} M, 72 hr at 37°C). The inset shows comparative EGF binding in BALB/c and SENCAR primary epidermal basal cells. All values are a percentage of "no addition" values. TPA treatment was 10 ng/mL, 2.5 hr, and retinoic acid treatment was as above. The binding assay was incubated for 6 hr at 0°C. Total cell-associated radioactivity values were normalized to controls.

Table 2. Retroviruses and mouse epidermal cells: expression and infection.

	BALB/c	SENCAR
Expression of endogenous infectious retrovirus		
In primary cells	+	–
In cell lines resistant to Ca^{2+}-induced terminal differentiation	+	+
Presence of receptors for virus gp 70	+	+
Infectibility by R-MuLV and AKR MuLV	+	+

ment. This finding implies that resistance may be related to poor responsiveness to TPA, the commonly used promoter, and, conversely, sensitivity may be a consequence of high responsiveness to TPA. A recent report (*17*) suggests that resistance or sensitivity to initiation-promotion may vary depending on the promoting agent used.

Recent studies in our laboratory have provided strong support for a relationship between initiation and resistance to Ca^{2+}-induced terminal differentiation (*18*). In direct comparisons of the yield of Ca^{2+}-resistant foci from BALB/c and SENCAR epidermal cells initiated either *in vivo* or in culture by DMBA or MNNG, little, if any, difference between strains in number of foci obtained for a given treatment has been found. Notable was the frequent presence of foci (rarely present in BALB/c cells) in untreated dishes of SENCAR cells, consistent with the formation of tumors in SENCAR mice treated with TPA alone in the absence of exogenous initiation. In spite of the absence of quantitative strain differences in carcinogen-induced, Ca^{2+}-resistant foci, there are qualitative differences that may provide useful clues, but these differences have been difficult to quantify. Although some SENCAR foci resemble those seen in BALB/c cultures, others have a different appearance and behavior. In general, it has been much easier to obtain cell lines from SENCAR than from BALB/c foci due to differences in survival after subculture. The inducibility of ornithine decarboxylase by TPA and transglutaminase by retinoic acid was generally greater in cell lines derived from SENCAR than from BALB/c foci. A detailed comparison of properties of these cell lines is in progress (Kawamura et al., in preparation). The resistant cell lines obtained from SENCAR foci differ in some responses to TPA in culture from cell lines derived from papillomas on SENCAR mice. TPA treatment of papilloma cell lines, but not all Ca^{2+}-resistant lines, leads to stimulation of DNA

synthesis and enhanced cloning efficiency (Yuspa et al., in preparation).

In conclusion, the biological and biochemical responses of SENCAR and BALB/c primary epidermal cells in culture in a variety of areas, including DNA repair, immune functions, growth in the presence of modulators of carcinogenesis, and binding and metabolism of EGF, have been remarkably similar. We have yet to determine whether these similarities will persist if cultured cells are subjected to repeated challenge with TPA in a manner similar to carcinogenesis protocols *in vivo*. The low levels of spontaneous expression of infectious xenotropic virus by BALB/c but not SENCAR primary cells is probably of no consequence because of the long periods (4 to 6 weeks) of cocultivation necessary to detect the difference. Oncogene expression was not significantly different in BALB/c and SENCAR cells. Although evidence from both *in vivo* and *in vitro* studies points to the existence of a population of constitutively initiated epidermal cells in SENCAR that is absent or greatly reduced in BALB/c skin, the role of such cells in SENCAR sensitivity has not been well defined. However, we have provided evidence that in response to the exogenous chemical initiators MNNG and DMBA, BALB/c cells are quantitatively equally as well initiated as SENCAR cells. We are currently characterizing what we believe are initiated cells, especially with reference to their response to promoters and their potential for conversion to malignancy. These cells and their response to promoters may provide the key to SENCAR sensitivity.

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